TITLE OF THE INVENTION

SECRETED CHLAMYDIA POLYPEPTIDES, POLYNUCLEOTIDES CODING THEREFOR, THERAPEUTIC AND DIAGNOSTIC USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Application 60/448,879, filed on February 24, 2003; the entire contents of which are incorporated herein by reference.

Field of the Invention

The present invention relates to secreted *Chlamydia* polypeptides. More particularly, the present invention relates to secreted *Chlamydia* polypeptides expressed by a Gram-negative bacterial strain and secreted by the type III secretion pathway of said bacterial strain. The present invention also relates to the polynucleotides coding for these secreted *Chlamydia* polypeptides, as well as to the therapeutic, including vaccination, and diagnostic uses of these secreted *Chlamydia* polypeptides.

BACKGROUND OF THE INVENTION

Chlamydiae are gram-negative bacteria that proliferate only within eukaryotic host-cells. The three species pathogenic to humans, *Chlamydia trachomatis, Chlamydia psittaci* and *Chlamydia pneumoniae,* cause a number of diseases, including trachoma, sexually transmitted disease, pelvic inflammatory disease, respiratory diseases, such as bronchitis, pneumonia and their sequelae (Gregory, D.W. and W. Schaffner. (1997). Psittacosis. *Seminars in Respiratory Infections.* **12:** 7-11; Kuo, C.-C., L. Jackson, L. Campbell, and J. Grayston. (1995). *Chlamydia pneumoniae (TWAR). Clin. Microbiol. Rev.* **8:** 451-61; Stamm, W.E. (1999). Chlamydia trachomatis infections: progress and problems. *J. Infect. Dis.* **179**: S380-3). In addition, *Chlamydia psittaci* and *Chlamydia pecorum* have been demonstrated to be pathogenic to animals other than humans, and as such have a significant impact on agricultural economics. In particular, *Chlamydia psittaci* and *Chlamydia pecorum* have been causatively linked to abortion and pneumonitis in cattle, pig, and sheep (Fukushi et al. (1993). *Microbiol Immunol.* **37**(7): 516-522; Tanner et al. (1999). *Chlamydia: Intercellular Biology, Pathogenesis, and Immunity.* Chapter 1).

During its cycle, *Chlamydia* adopts two distinct morphologies: a small infectious form, the elementary body (EB, 0.3 μm in diameter), and a larger replicative form, the reticulate body (RB, 1 μm in diameter) (Moulder, J.W. (1991). Interaction of chlamydiae and host cells in vitro. *Microbiol. Rev.* **55:** 143-90.). EBs are adapted for survival in the extracellular space: their outer membrane is made rigid by a network of disulfide bonds and the bacteria are metabolically inactive. They initiate infection by attaching to a suitable host cell, principally epithelial cells of the mucosa, and they are internalized by a mechanism resembling phagocytosis (Boleti, H., A. Benmerah, D. Ojcius, N. Cerf-Bensussan, and A. Dautry-Varsat. (1999). *Chlamydia* infection of epithelial cells expressing dynamin and Eps15 mutants: clathrin-independent entry into cells and dynamin-dependent productive growth. J. *Cell. Sci.* **112**: 1487-1496). Within a few hours after internalization, EBs differentiate into replicative RBs, which proliferate in a growing vacuole and produce up to about a thousand progeny. After 2 to 3 days of infection RBs differentiate back into EBs, which are delivered to the extracellular space, and a new infectious cycle can begin.

Throughout their cycle in the host cell, Chlamydiae remain in a membrane-bound compartment called an inclusion. Bacteria escape from host defense mechanisms (i.e., cytosolic proteases or lysosomal enzymes) by preventing fusion of the inclusion with acidic degradative compartments of host cells. In addition, most bacterial antigens avoid detection by the host immune system, since these antigens do not access the infected cell plasma membrane. Inhibition of phagolysosome fusion is limited to *Chlamydia psittaci*-laden vacuoles. (Eissenberg, L.G., and P.B. Wyrick. (1981) *Infect. Immun.* **32:** 889-896; Friis, R.R. (1972). Interaction of L cells and *Chlamydia psittaci*: entry of the parasite and host responses to its development. *J. Bacteriol.* **110**: 706-721; Heinzen, R.A., M.A. Scidmore, D.D. Rockey, and T. Hackstadt. (1996). Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetii and Chlamydia trachomatis. Infect. Immun.* **64:** 769-809; Schramm, N., C.R. Bagnell, and P.B. Wyrick. (1996). Vesicles containing *Chlamydia trachomatis* serovar L2 remain above pH 6 within HEC-1B cells. *Infect. Immun.* **64**:1208-1214). In fact, Chlamydiae seem to exchange very little with the endocytic compartments (Taraska, T., D.M. Ward, R.S. Ajioka, P.B. Wyrick, S.R. Davis-Kaplan, C.H. Davis, and J. Kaplan. (1996). The late chlamydial inclusion membrane is not derived from the endocytic pathway and is relatively deficient in host proteins. *Infect. Immun.* **64:** 3713-372; Van Ooij, C., G. Apodaca, and J. Engel. (1997). Characterization of the *Chlamydia trachomatis* vacuole and its interaction with the host endocytic pathway in HeLa cells. *Infect. Immun.* **65:** 758-766).

Yet, during their development cycle, the volume of the inclusions increases considerably, until they occupy a large portion of the cytosol. Part of the lipids necessary for this growth come from the host cell (Hatch, G.M., and G. McClarty. (1998). Phospholipid composition of purified Chlamydia trachomatis mimics that of the eucaryotic host cell. *Infection & Immunity.* **66**: 3727-35). Sphingomyelin, synthesized in the Golgi apparatus, is transported to the inclusion membrane and incorporated into bacteria (Hackstadt, T., M.A. Scidmore, and D.D. Rockey. (1995). Lipid metabolism in *Chlamydia trachomatis*- infected cells: directed trafficking of Golgi-derived sphingolipids to the chlamydial inclusion. *Proc. Natl. Acad. Sci, USA.* **92**: 4877-4881).

Surprisingly, no proteins of eukaryotic origin have been found associated with the inclusion. However, a number of prokaryotic proteins have been localized on the membrane of the inclusion, by fluorescence microscopy using antibodies generated against a variety of chlamydial proteins (Bannantine, J.P., R.S. Griffiths, W. Viratyosin, W.J. Brown, and D.D. Rockey. (2000). A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. *Cell. Microbiol. 2:* 35-47; Bannantine, J.P., D.D. Rockey, and T. Hackstadt. (1998). Tandem genes of Chlamydia psittaci that encode proteins located to the inclusion membrane. *Mol. Microbiol.* **28**: 1017-26; Rockey, D.D., R.A. Heinzen, and T. Hackstadt. (1995). Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. *Mol. Microbiol.* **15:** 617-626; Scidmore-Carlson, M.A., E.I. Shaw, C.A. Dooley, E.R. Fischer, and T. Hackstadt. (1999). Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. *Mol. Microbiol.* **33:** 753-765). These proteins show no sequence similarities but have in common the presence of a large (40 to 70 amino acids) hydrophobic region and have therefore been grouped into a structural family called the Inc family.

The three first Inc proteins identified, IncA, IncB and IncC, are probably major components of the inclusion since they are recognized by antisera from convalescent guinea pigs infected with *C. psittaci* (Bannantine, J., W. Stamm, R. Suchland, and D. Rockey. (1998). *Chlamydia trachomatis* IncA is localized to the inclusion membrane and is recognized by antisera from infected humans and primates. *Infect. Immun.* **66**: 6017-6021; Rockey, D.D., R.A. Heinzen, and T. Hackstadt. (1995). Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. *Mol. Microbiol.* **15:** 617-626). They have homologs encoded by *C. trachomatis and C.*

pneumoniae genomes, exhibiting about 20% global sequence identity and up to 50% sequence identity in the regions of highest similarity.

Inc proteins have been grouped into a family on two criteria: they have a large (larger than 40 residues) hydrophobic domain and they localize to the membrane of the inclusion in the host cell. The demonstration that several proteins encoded by the *C. trachomatis* genome and one protein from *C. pneumoniae* belong to this family was based on their localization to the membrane of the inclusion by immunofluorescence using specific antibodies (Bannantine, J., W. Stamm, R. Suchland, and D. Rockey. (1998). *Chlamydia trachomatis* IncA is localized to the inclusion membrane and is recognized by antisera from infected humans and primates. *Infect. Immun.* **66**: 6017-6021;Bannantine, J.P., R.S. Griffiths, W. Viratyosin, W.J. Brown, and D.D. Rockey. (2000). A secondary structure motif predictive of protein localization to the chlamydial inclusion membrane. *Cell. Microbiol. 2:* 35-47; Bannantine, J.P., D.D. Rockey, and T. Hackstadt. (1998). Tandem genes of Chlamydia psittaci that encode proteins located to the inclusion membrane. *Mol. Microbiol.* **28**: 1017-26; Rockey, D.D., R.A. Heinzen, and T. Hackstadt. (1995). Cloning and characterization of a *Chlamydia psittaci* gene coding for a protein localized in the inclusion membrane of infected cells. *Mol. Microbiol.* **15:** 617-626.; Scidmore-Carlson, M.A., E.I. Shaw, C.A. Dooley, E.R. Fischer, and T. Hackstadt. (1999). Identification and characterization of a *Chlamydia trachomatis* early operon encoding four novel inclusion membrane proteins. *Mol. Microbiol.* **33:** 753-765). However, the mechanism of their secretion and insertion into the membrane of the inclusion had not been investigated yet, due to the lack of genetic tools for members of *Chlamydia* spp.

How do chlamydial proteins reach the membrane of the inclusion? Four pathways of protein secretion (types I to IV) have been described in Gram-negative bacteria. Type III secretion machines allow for the translocation of bacterial effectors into the eukaryotic cell cytosol during bacterial contact with the cell surface (Hueck, C. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* **62:** 379-433). The inventors hypothesized that Chlamydiae use type III secretion to insert proteins into the membrane of the inclusion (Subtil A., A. Blocker, and A. Dautry-Varsat. (2000). Type III secretion system in *Chlamydia:* identified members and candidates. *Microbes Infect.* **2**: 367-369). Presence of genes encoding putative components of a type III secretion machinery in the *Chlamydia* genome suggested that Inc proteins might be secreted by a type III secretion pathway since type III secretion is used by a large number of Gram-negative pathogens for the insertion or the translocation of bacterial proteins into or through

eukaryotic cell membranes. In the absence of genetic tools to manipulate the *Chlamydia* genome, it was difficult to test this hypothesis directly. *Shigella* uses a type III secretion system for entry into epithelial cells and for dissemination (Nhieu, G.T., and P.J. Sansonetti. (1999). Mechanism of Shigella entry into epithelial cells. *Curr. Opin. Microbiol.* **2:** 51-5; Page, A.-L., H. Ohayon, P.J. Sansonetti, and C. Parsot. (1999). The secreted IpaB and IpaC invasins and their cytoplasmic chaperone IpgC are required for intercellular dissemination of *Shigella flexneri. Cell. Microbiol.* **1***:* 183-193; Schuch, R., R.C. Sandlin, and A.T. Maurelli. (1999). A system for identifying post-invasion functions of invasion genes: requirements for the Mxi-Spa type III secretion pathway of Shigella flexneri in intercellular dissemination, *Mol. Microbiol.* **34**:675-689). The secretion signal involved in secretion of proteins by the type III secretion pathway is not known. However, it is often located either within the first fifteen codons of the mRNA coding for the secreted proteins, or within the N-terminal part of the secreted protein (Anderson, D.M., and O. Schneewind. (1999). Type III machines of Gram-negative pathogens: injecting virulence factors into host cells and more. *Curr. Opin. Microbiol.* **2**: 18-24). Although the genomes of several *Chlamydia* species are now available, it is not possible to identify secreted proteins on the basis of their amino acid sequence.

Genes coding for the type III secretion system in *Shigella* are known (Hueck, C. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* **62:** 379-433) and it has been hypothesized that Inc proteins may be secreted by a similar machinery.

Expression of heterologous proteins by use of type III machinery of *Shigella has* already been obtained (see "Functional conservation of the *Salmonella* and *Shigella* effectors of entry into epithelial cells" published in *Molecular Microbiology* (1995) 17 (4), 781-789, Hermant, Ménard, Arricau, Parsot and Popoff). However, as indicated in the title, this document relates to *Salmonella* proteins, which is different from the *Chlamydia* proteins due to the phylogenic distance of *Chlamydia* from other organisms (see page 369, first column of Subtil, *et al.*; *Microbes and Infection* 2, 2000; Subtil A., A. Blocker, and A. Dautry-Varsat. (2000). Type III secretion system in *Chlamydia*: identified members and candidates. *Microbes Infect.* **2**: 367-369).

Furthermore, methods for screening inhibitors and activators of type III secretion machinery in gram-negative bacteria, in *Shigella,* have already been disclosed in WO 99/58714.

Several gram-negative pathogens, including chlamydiae, possess a type III secretion machinery for the insertion or the translocation of bacterial proteins into or through

eukaryotic membranes. In U.S. Patent Application 10/014,670 (the entire disclosure of which is incorporated herein by reference), the present inventors constructed chimeras by fusing the N-terminal part of these proteins with a reporter gene, the Cya protein of *Bordetella pertussis*, and expressed these chimeras in various strains of *Shigella flexneri*. The use of a reporter gene is well known by those of ordinary skill in the art. The reporter sequence, which codes for an easily detectable protein, is linked to a fragment of the DNA of interest. The use of the *B. pertussis cya* gene as a reporter gene, chosen in the present invention, is reported by: Jones *et al.*, 1998 and Sory and Cornelis, 1994 (Jones, M.A., M.W. Wood, P.B. Mullan, P.R. Watson, T.S. Wallis, and E.E. Galyov. (1998). Secreted effector proteins of Salmonella dublin act in concert to induce enteritis. *Infection & Immunity.* **66:** 5799-804; Sory, M.P., and G.R. Cornelis. (1994). Translocation of a hybrid YopE-adenylate cyclase from Yersinia enterocolitica into HeLa cells. *Mol. Microbiol.* **14**: 583-94). Moreover, the inventors have shown that several chlamydial proteins, including members of the Inc family and proteins selected for a hydropathic profile similar to that of Inc proteins, are secreted by the type III secretion machinery of *S. flexneri*. Identification of such proteins is very important since these proteins may be involved in functions essential for the intracellular survival of *Chlamydia*. They may serve as a basis for the development of a chlamydiae vaccine or a treatment against *Chlamydia* infection, by suggesting non-immune therapeutic interventions capable of inhibiting bacterial development, as well as for the development of Chlamydia infection diagnosis.

A need continues to exist, however, for further elucidation of chlamydial polypeptides that are secreted, as well as the diagnostic and therapeutic uses of the same.

In the present invention, the inventors describe the identification of several new proteins secreted by *Chlamydia spp.* during infection. Most of these proteins are from the human pathogens *C. trachomatis* and *C. pneumoniae*, and a few proteins are from one strain of the human and animal pathogen *C. psittaci*. In several cases the inventors have shown that if a protein is secreted in one species, homologous proteins from other species are also secreted (see below, category 1). Therefore knowledge that a protein is secreted in one species gives a good indication that it is also true in another species and this can be checked with the method described herein. Thus, the results presented here can also be extended to different *Chlamydia* species, to be used as a basis for the development of a chlamydiae vaccine or a treatment against *Chlamydia* infection, as well as for the development of Chlamydia infection diagnosis, in humans or in animals.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a purified secreted polypeptide from *Chlamydia* spp., e.g., *Chlamydia pneumoniae, Chlamydia trachomatis, Chlamydia pecorum,* or *Chlamydia psittaci.* The purified polypeptide may be selected by a method comprising (a) providing a recombinant expression vector containing at least a polynucleotide coding for the polypeptide of interest; (b) transforming a Gram-negative strain containing a type III secretion pathway with said recombinant vector; (c) expressing this vector in the Gram-negative strain transformed in (b); and (d) detecting the secretion of said polynucleotide expression product; wherein the secretion of said expression product indicates that it corresponds to a secreted *Chlamydia* polypeptide. The detection of the secretion of expression product is made by detecting the presence of said product outside the bacteria.

Alternatively, the purified polypeptide may be selected by a method comprising (a) providing a recombinant expression vector comprising at least a DNA coding for the polypeptide of interest fused to a reporter gene (i.e., the recombinant expression vector comprises a fusion construct in which one component of the construct is the polynucleotide of interest and a second component of the construct is the polynucleotide sequence of a reporter gene); (b) transforming a Gram-negative strain containing a type III secretion pathway with said recombinant vector; (c) expressing this vector in the Gram-negative strain transformed in (b); and (d) detecting the secretion of said reporter gene expression product; wherein the secretion of said expression product indicates that the fused DNA (i.e., the fusion construct of (a), which contains the polynucleotide of interest and a polynucleotide sequence of a reporter gene) contains at least a polynucleotide corresponding to a secreted *Chlamydia* polypeptide. The detection of the secretion of expression product is performed by detecting the presence of said product outside the bacteria.

Another object of the present invention is to provide a purified polynucleotide coding for at least one polypeptide that is secreted from *Chlamydia.*

Another object of the present invention is to provide an immunogenic composition comprising a secreted *Chlamydia* polypeptide, or an immunogenic fragment thereof.

Another object of the present invention is to provide a vaccinating composition against *Chlamydia* infection comprising a secreted *Chlamydia* polypeptide, or an immunogenic fragment thereof. Said composition is a human and/or a veterinary vaccinating composition. For example, said infection is a sexually transmitted disease , respiratory disease, such as pneumonia or bronchitis, or contributes to atherosclerosis.

Another object of the present invention is to provide a therapeutic composition active against *Chlamydia* infection comprising a molecule, which inhibits the secretion of a secreted *Chlamydia* polypeptide.

Another object of the present invention is to provide a complex comprising a secreted *Chlamydia* polypeptide and an antibody directed against said polypeptide.

Another object of the present invention is to provide an antibody against *Chlamydia* wherein the antibody is directed against a secreted *Chlamydia* polypeptide, or an immunogenic fragment thereof, wherein said antibody is a monoclonal antibody or a polyclonal antibody.

Another object of the present invention is to provide methods for diagnosing a *Chlamydia* infection in an animal, including a human. A method for diagnosing a *Chlamydia* infection in an animal, comprises (a) providing a secreted polypeptide of *Chlamydia*, or an immunogenic fragment thereof, optionally labeled; (b) bringing said polypeptide or immunogenic fragment thereof into contact with a serum sample of said animal; and (c) detecting complexes formed between said polypeptide or immunogenic fragment thereof and antibodies contained in the serum sample; wherein said complexes are indicative of a *Chlamydia* infection in said animal.

Alternatively, a method for diagnosing a *Chlamydia* infection in an animal, including a human, comprises (a) providing an animal sample of a tissue suspected to be infected by *Chlamydia;* (b) bringing said sample into contact with an antibody against *Chlamydia* wherein said antibody is directed against a purified secreted polypeptide of *Chlamydia*; and (c) detecting antigen-antibody complexion; wherein said complexion is indicative of a *Chlamydia* infection in said animal.

Within the objects of the present invention, said purified polypeptides of *Chlamydia* may be identified by their expression and secretion in a Gram negative strain containing a type III secretion pathway.

In yet another object, the present invention provides a method of detecting *Chlamydia* in an animal, including a human, using a probe or primer pair selected from Table I or II. The method for detecting *Chlamydia* in an animal comprises (a) providing an animal sample of a tissue suspected to be infected by *Chlamydia;* (b) adding a primer pair selected from the list presented in Tables I and II to the tissue sample; (c) amplifying a polynucleotide that encodes for the protein which corresponds to the primer pair selected; and (d) detecting the presence of Chlamydia by the presence or absence of said polynucleotide.

In still another object of the present invention is a method of detecting *Chlamydia* in an animal, including a human, using a probe that hybridizes under stringent conditions with a polynucleotide that encodes a polypeptide of the present invention. The method for detecting *Chlamydia* in an animal comprises (a) providing an animal sample of a tissue suspected to be infected by *Chlamydia;* (b) adding a hybridization probe that hybridizes with a polynucleotide that encodes a polypeptide of the present invention under stringent conditions; and (c) detecting the presence of Chlamydia by the presence or absence of said polynucleotide.

In still another object of the present invention is a method of detecting *Chlamydia* in an animal, including a human, using an antibody, optionally labeled, that forms a complex with a polypeptide of the present invention. The method for detecting *Chlamydia* in an animal comprises (a) providing an animal sample of a tissue suspected to be infected by *Chlamydia;* (b) adding an antibody, optionally labeled, that forms a complex with a polypeptide of the present invention under conditions suitable for complex formation; and (c) detecting the presence of Chlamydia by the presence or absence of said polypeptide.

Another object of the present invention is to provide a recombinant plasmid for the expression of a secreted *Chlamydia* polypeptide.

Another object of the present invention is to provide a recombinant prokaryotic cell, for example a recombinant Gram-negative bacterial strain, transformed by a vector comprising at least a polynucleotide encoding a secreted *Chlamydia* polypeptide.

Another object of the present invention is to provide a method of preventing or treating a *Chlamydia* infection in an animal, including a human, which comprises administering an effective amount of a purified secreted polypeptide, or immunogenic fragment thereof, of *Chlamydia* of the present invention.

In still another object, the present invention provides a method of preventing or treating a *Chlamydia* infection in an animal, which comprises administering an effective amount of an antibody directed against a secreted *Chlamydia* polypeptide according to the present invention, or an immunogenic fragment thereof, to an animal in need thereof.

Still another object of the present invention is to provide a method of screening for an active molecule inhibiting the secretion of a secreted *Chlamydia* polypeptide, comprising (a) supplying an active molecule to a culture of *Chlamydia*; (b) growing or incubating said culture for a time and under conditions suitable for said active molecule to exert an activity upon said culture; (c) adding a primer pair for a *Chlamydia* polypeptide to the culture; (d) amplifying a polynucleotide that encodes for the polypeptide which corresponds to the primer

pair selected; and (e) detecting the presence of the secreted *Chlamydia* polypeptide by the presence or absence of said polynucleotide.

A further object of the present invention is to provide a method of detecting the presence of a *Chlamydia* polypeptide by using a probe that hybridizes under stringent conditions with a polynucleotide that encodes the polypeptide or by using antibodies, optionally labeled, that form a complex with the polypeptide.

In yet another object of the present invention is to provide a method of screening for an active molecule inhibiting the secretion of a secreted *Chlamydia* polypeptide, comprising (a) supplying an active molecule to a culture of *Chlamydia*; (b) growing or incubating said culture for a time and under conditions suitable for said active molecule to exert an activity upon said culture; and (c) evaluating the presence of a polypeptide according to the present invention.

The above objects highlight certain aspects of the invention. Additional objects, aspects and embodiments of the invention are found in the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following Figures in conjunction with the detailed description below.

Figure 1: Illustration of the secretion assay in *Shigella*. The *ipaB* mutant strain of *Shigella* constitutively secretes high level of effectors by the type III pathway. This strain was transformed with different chimeric constructs and secretion of the chimeras was probed on colonies. Colonies expressing the different constructs were picked on a fresh LB plate in the morning and incubated for 8 hours at 37 °C. The colonies were then covered with a PVDF membrane and grown overnight at 37 °C. Secretion of the chimera was detected on this membrane by Western blot using anti-cyclase antibody. Secreted proteins formed a halo around the colonies (2 lower rows) while non-secreted proteins were detected only on the spot where the colonies had grown (2 upper rows).

Figure 2: Full length chlamydial proteins are secreted by *Shigella*. The ipaB mutant strain of *Shigella* constitutively secretes a high level of effectors by the type III pathway, while the *mxiD* mutant strain is totally deficient for type III secretion. In this experiment, the strains were transformed with Psi1058 and the presence of the protein in the culture supernatant was probed by Western blot. Psi1058 was found in the supernatant (S) fraction

of the *ipaB* stain culture. In the *mxiD* strain it was found only associated with the pellet (P) fraction. This result shows that Psi1058 is secreted by a type III mechanism in *S. flexneri*. The same result was found with *S. flexneri* transformed with CPn0175, Psi705, Psi725, Psi774, and Psi1005.

Figure 3: HeLa cells were infected or not for 24 hours with *C.psittaci* GPIC strain, scrapped and broken by 7 passages through a $25G1^{1/2}$ syringe needle in homogenization buffer (0.25 M sucrose, 0.1% gelatine, 0.5 mM EGTA, 3 mM imidazole pH 7.4). The cells were centrifuged for 15 minutes at 800 g, the pellet was resuspended in gel sample buffer (pellet 1 contains unbroken cells, bacteria and cell nuclei) and the supernatant was centrifuged for 45 minutes at 541 000 g (pellet 2 contains cellular membranes and bacteria, the supernatant contains proteins present in the cytosol). Equal fractions of pellets and supernatant were analyzed by electrophoresis in 8% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE). After electrophoresis, proteins were transferred on a PVDF membrane (Millipore Corporation, Bedford, MA), and the membrane was used for blotting with anti-Psi0705 antibody. Western blotting and revelation were performed by enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Psi0705 was present in the pellet fractions as well as in the supernatant fraction (cytosolic material). As a control MOMP (major outer membrane protein) was present only in the pellet fractions.

Figure 4: HeLa cells were infected for 24 hours with *C.psittaci* GPIC strain, fixed and permeablized with 0.05% saponin. Psi0710 was labeled with specific rabbit antibodies raised against the purified protein, followed by anti-rabbit Alexa488-coupled secondary antibodies (Molecular Probes). Cells were examined under an epifluorescence microscope attached to a cooled CCD-camera. Arrowheads point to fibers extending from the membrane of inclusion, to which Psi0710 is associated.

DETAILED DESCRIPTION OF THE INVENTION

Unless specifically defined, all technical and scientific terms used herein have the same meaning as commonly understood by a skilled artisan in biochemistry, molecular biology, enzymology, genetics, immunology, general medicine and veterinary medicine.

All methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, with suitable methods and materials being described herein. All publications, patent applications, patents, and other references

mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. Further, the materials, methods, and examples are illustrative only and are not intended to be limiting, unless otherwise specified.

A "polypeptide" as used herein is understood to mean a sequence of several amino acid residues linked by peptide bonds. Such amino acids are known in the art and encompass the unmodified and modified amino acids. In addition, one or more modifications known in the art such as glycosylation, phosphorylation, etc may modify the polypeptide.

The term "secreted *Chlamydia* polypeptide" as used herein is understood to mean a *Chlamydia* protein, or fragment of the protein, comprising at least 20 amino acids, detectable outside the bacteria.

The term "homologous" as used herein is understood to mean two or more proteins of *Chlamydia* from the same species or from a different species. Within the meaning of this term, said two or more proteins share at least 40% identity to a fragment having at least 50 amino acids. Preferably, homologous *Chlamydia* proteins share at least 50% identity to a fragment having at least 50 amino acids. More preferably, homologous *Chlamydia* proteins share at least 60% identity, at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, or 100% identity to a fragment having at least 50 amino acids. Accordingly, homologous *Chlamydia* proteins are included within the scope of the present invention.

The term "outside the bacteria" as used herein is understood to mean that a certain portion of the polypeptide of the invention produced by the bacteria has crossed the inner membrane, the periplasm and the outer membrane of the bacteria and is either still bound to the outer membrane, found in the extracellular medium or found inside the host cell.

As used herein, the term "active molecule inhibiting the secretion of a secreted *Chlamydia* polypeptide" is understood to mean a molecule able to reduce, including to totally remove, said secretion by any means. For example, said reduction, including total removal, of secretion may result from an action of said molecule on the type III secretion system or on the expression of said polypeptide by the bacteria.

The term "type III secretion pathway" as used herein is understood to mean a complex association of various molecules, which are necessary for secretion of a polypeptide in a host in which it is normally expressed, as described in Hueck CJ. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev.* **62**: 379-433.

The term "immunogenic fragment" as it relates to polypeptides is understood to mean a polypeptide fragment of at least 6 amino acids and preferably at least 10 amino acids sufficient to induce an immune response when it is administered to a host eukaryotic organism.

The term "heterologous" as it relates to protein or polypeptide secretion systems is understood to mean that the protein or polypeptide is not normally expressed in a host.

Nucleic acids can be detected utilizing a nucleic acid amplification technique, such as polymerase chain reaction (PCR). Alternatively, the nucleic acid can be detected utilizing direct hybridization or by utilizing a restriction fragment length polymorphism.

Hybridization protocols are known in the art and are disclosed, for example, in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989).

The term "*Chlamydia* infection" as used herein means an infection in an animal, especially a human, that is caused by a species of *Chlamydia*; i.e., *Chlamydia trachomatis, Chlamydia psittaci, Chlamydia pneumoniae* and *Chlamydia pecorum.* Within the context of the present invention, a *Chlamydia* infection causes a number of diseases, including trachoma, sexually transmitted disease, pelvic inflammatory disease, respiratory diseases, such as bronchitis, pneumonia and their sequelae, such as atherosclerosis.

As used herein, stringent hybridization conditions are those conditions which allow hybridization between polynucleotides that are 75%, 80%, 85%, 90%, 95%, or 98% homologous as determined using conventional homology programs, an example of which is UWGCG sequence analysis program available from the University of Wisconsin. (Devereaux *et al.,* Nucl. Acids Res. 12: 387-397 (1984)). Such stringent hybridization conditions typically include washing the hybridization reaction mixture in 2X SSC and 0.5% SDS at 65°C (Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989)). Of course, one of skill in the art will recognize that conditions can be varied depending on the length of the polynucleotides to be hybridized and the GC content of the polynucleotides see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989).

In this invention "primer" or "probe" means a polynucleotide, especially an oligonuclotide, which is produced synthetically or biologically and includes a specific nucleotide sequence, which permits hybridization to a section containing the target nucleotide sequence. Also in the present invention, the phrase "primer pair selected from the list

presented in Tables I and II" means a single forward primer and the corresponding reverse primer for the protein of interest.

Defined primers or probes, as well as all other oligonucleotides and polynuclotides of the present invention, may be produced by any of several well-known methods, including automated solid-phase chemical synthesis using cyanoethyl-phosphoramidite precursors. Other well-known methods for construction of synthetic primers/oligonucleotides may, of course, be employed. J. Sambrook, E. F. Fritsch and T. Maniatis, Molecular Cloning 11 (2d ed. 1989).

The primers used to amplify the sample nucleic acids may be coupled to a detectable moiety. A preferred example of such a detectable moiety is fluorescein, which is a standard label used in nucleic acid sequencing systems using laser light as a detection system. Other detectable labels can also be employed, however, including other fluorophores, radio labels, chemical couplers such as biotin which can be detected with streptavidin-linked enzymes, and epitope tags such as digoxigenin detected using antibodies. The primers may be modified whereby another nucleotide is added to, removed from, or substituted for at least one nucleotide in the oligonucleotide. Introduction of known labels such as radioactive substances, enzymes, fluorescence substances, etc. after synthesis of oligonucleotide is also included therein.

Similarly, the probes/oligonucleotides used to hybridize with the polynucleotides coding for the polypeptides of the invention, for example for the purpose of detection of such a polynucleotide, may be coupled to a detectable moiety.

Examples of suitable primers for use in the present invention are shown in Table I.

The inventors have constructed chimeras between the N-terminal domain of several chlamydial proteins and a reporter protein, the calmodulin-dependent adenylate cyclase (Cya) from *B. pertussis,* and have tested whether these chimeras were secreted by *S. flexneri.* Proteins analyzed in this study can be classified into five categories.

(i) Corresponding chimeras from *C. pneumoniae, C. trachomatis,* and *C. psittaci* that are homologous and are secreted. Sequence analysis has shown that these three species are rather distant, and, as a consequence, the percentage of amino acid identity of the N-terminal domain between homologous proteins is generally low. Consequently, the chimeras constructed from homologous proteins share little identity in their amino terminal region, which is the region containing the putative secretion signal. Therefore, the identification that the three homologous chimeras (one from

each of the *Chlamydia* species above) are secreted strongly supports that the corresponding proteins are secreted during *Chlamydia* infection. This category comprises the following polypeptides: CPn0330 ; CT083 ; Psi0330 ; CPn0379 ; CT053 ; Psi0379 ; CPn0595 ; CT476 ; Psi0595 ; CPn0648 ; CT529 ; Psi0648 ; CPn 0671 ; CT550 ; Psi0671 ; CPn0710 ; CT666 ; Psi0710 ; CPn0761 ; CT610 ; Psi0761 ; CPn0774 ; CT606.1 ; Psi0774 ; CPn1002 ; CT845 ; Psi1002 ; CPn1005 ; CT848 ; Psi1005 ; CPn1022 ; CT863 ; Psi1022.

(ii) Proteins of *C. pneumoniae* and *C. trachomatis* which are homologous and for which the 2 corresponding chimeras are secreted. For the same reasons as explained above in the case of category 1, these results support that these proteins are also secreted during *Chlamydia* infection. This category comprises the following polypeptides: CPn0490 ; CT387 ; CPn0705 ; CT671 ; CPn0711 ; CT665 ; CPn0712 ; CT664 ; CPn0725 ; CT652.1 ; CPn0770 ; CT642 ; CPn0859 ; CT718 ; CPn0906 ; CT763.

(iii) *C. pneumoniae* proteins that are secreted, which have no homolog in *C. trachomatis* or *C. psittaci*. They may be important proteins, being specific of *C. pneumoniae*. This category comprises the following polypeptides: CPn0009 ; CPn0012 ; CPn0028 ; CPn0049; CPn0063 ; CPn0066 ; CPn0067 ; CPn0130 ; CPn0132 ; CPn0146 ; CPn0167 ; CPn0174 ; CPn0175 ; CPn0181 ; CPn0210; CPn0211 ; CPn0220 ; CPn0223 ; CPn0226 ; CPn0243 ; CPn0267 ; CPn0277 ; CPn0284 ; CPn0357 ; CPn0365 ; CPn0585 ; CPn0829 ; CPn1027.

(iv) *C. pneumoniae* proteins that have a homolog in at least one of the other 2 sequenced *Chlamydia* species and for which secretion of the corresponding homologous chimera is undetermined. This category comprises the following polypeptides: CPn0026 ; CPn0104 ; CPn0186 ; CPn0206 ; CPn0291 ; CPn0292 ; CPn0405 ; CPn0443 ; CPn0480 ; CPn0489 ; CPn0556 ; CPn0673 ; CPn0681 ; CPn0720 ; CPn0746 ; CPn0853 ; CPn0879 ; CPn0939 ; CPn1019 ; CPn1020 ; CPn1032.

(v) *C. pneumoniae* proteins that have homologs in the other 2 sequenced *Chlamydia* species and for which secretion of the corresponding homologous chimeras are negative or undetermined. Although these results support that most proteins in this category are secreted by *Chlamydia*

during infection, the Inventors regard them as somewhat less strong candidates than the proteins classified in the other 4 categories, until more data are obtained (see for example the result with Psi1058 below). This category comprises the following polypeptides: CPn0105 ; CPn0287 ; CPn0334 ; CPn0374 ; CPn0399 ; CPn0497 ; CPn0522 ; CPn0582 ; CPn0588 ; CPn0729 ; CPn0755 ; CPn0764 ; CPn0792 ; CPn0820 ; CPn0821 ; CPn1007 ; CPn1016 ; CPn1058.

The polypeptides of the invention are administered in a dose, which is effective to vaccinate an animal, preferably a human, against *Chlamydial* infection or to treat an animal, preferably a human, having a *Chlamydial* infection. As used herein, an effective amount of the polypeptides to achieve this goal is generally from about 2 ng to 2 mg/kg of body weight per week, preferably about 2 μg/kg per week. This range includes all specific values and subranges therebetween.

In a preferred embodiment, the *Chlamydia* polypeptide is homologous to one or more *Chlamydia pneumoniae* proteins selected from the group consisting of CPn0104 (SEQ ID NO: 2), CPn0206 (SEQ ID NO: 4), CPn0210 (SEQ ID NO: 6), CPn0399 (SEQ ID NO: 8), CPn0405 (SEQ ID NO: 10), CPn0443 (SEQ ID NO: 12), CPn0480 (SEQ ID NO: 14), CPn0489 (SEQ ID NO: 16), CPn0490 (SEQ ID NO: 18), CPn0497 (SEQ ID NO: 20), CPn0522 (SEQ ID NO: 22), CPn0556 (SEQ ID NO: 24), CPn0582 (SEQ ID NO: 26), CPn0588 (SEQ ID NO: 28), CPn0595 (SEQ ID NO: 30), CPn0671 (SEQ ID NO: 32), CPn0673 (SEQ ID NO: 34), CPn0681 (SEQ ID NO: 36), CPn0712 (SEQ ID NO: 38), CPn0720 (SEQ ID NO: 40), CPn0725 (SEQ ID NO: 42), CPn0729 (SEQ ID NO: 44), CPn0746 (SEQ ID NO: 46), CPn0755 (SEQ ID NO: 48), CPn0761 (SEQ ID NO: 50), CPn0764 (SEQ ID NO: 52), CPn0770 (SEQ ID NO: 54), CPn0774 (SEQ ID NO: 56), CPn0792 (SEQ ID NO: 58), CPn0853 (SEQ ID NO: 60), CPn0859 (SEQ ID NO: 62), CPn0879 (SEQ ID NO: 64), CPn0906 (SEQ ID NO: 66), CPn0939 (SEQ ID NO: 68), CPn1002 (SEQ ID NO: 70), CPn1005 (SEQ ID NO: 72), CPn1007 (SEQ ID NO: 74), CPn1019 (SEQ ID NO: 76), CPn1020 (SEQ ID NO: 78), CPn1032 (SEQ ID NO: 80), and CPn1058 (SEQ ID NO: 82); or a fragment thereof.

In another embodiment, the homologous *Chlamydia* polypeptide is a *Chlamydia trachomatis* protein selected from the group consisting of CT387 (SEQ ID NO: 84), CT476 (SEQ ID NO: 86), CT550 (SEQ ID NO: 88), CT606.1 (SEQ ID NO: 90), CT610 (SEQ ID NO: 92), CT642 (SEQ ID NO: 94), CT652.1 (SEQ ID NO: 96), CT664 (SEQ ID NO: 98),

CT718 (SEQ ID NO: 100), CT763 (SEQ ID NO: 102), CT845 (SEQ ID NO: 104), and CT848 (SEQ ID NO: 106); or a fragment thereof.

In still another embodiment, the homologous *Chlamydia* polypeptide is a *Chlamydia psittaci* protein selected from the group consisting of Psi0330 (SEQ ID NO: 108), Psi0379 (SEQ ID NO: 110), Psi0595 (SEQ ID NO: 112), Psi0648 (SEQ ID NO: 114), Psi0671 (SEQ ID NO: 116), Psi0705 (SEQ ID NO: 118), Psi0710 (SEQ ID NO: 120), Psi0761 (SEQ ID NO: 122), Psi0774 (SEQ ID NO: 124), Psi1002 (SEQ ID NO: 126), Psi1005 (SEQ ID NO: 128), Psi1022 (SEQ ID NO: 130), and Psi1058 (SEQ ID NO: 132); or a fragment thereof.

A *Chlamydia* infection in an animal, preferably a human, as used herein includes a number of diseases, including trachoma, sexually transmitted disease, pelvic inflammatory disease, respiratory diseases, such as bronchitis, pneumonia and their sequelae, such as atherosclerosis.

Examples of other animals envisioned within the purview of the present invention include: a horse (equine animal), a cow (bovine animal), a pig (porcine animal), a sheep (ovine animal), a goat (caprine animal), a bird, a dog, and a cat.

The polypeptides of the present invention may be administered as a pharmaceutical composition containing the polypeptide compound and a pharmaceutically acceptable carrier or diluent. The active materials can also be mixed with other active materials, which do not impair the desired action and/or supplement the desired action. Any route can administer the active materials according to the present invention, for example, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid or solid form.

For the purposes of parenteral therapeutic administration, the active ingredient may be incorporated into a solution or suspension. The solutions or suspensions may also include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Another mode of administration of the polypeptides of this invention is oral. Oral compositions will generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the aforesaid polypeptides may be incorporated with excipients and used in the form of tablets, gelatine capsules, troches, capsules, elixirs,

suspensions, syrups, wafers, chewing gums and the like. Compositions may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavoring agents, coloring agents and preserving agents. Tablets containing the active ingredient in admixture with nontoxic pharmaceutically acceptable excipients, which are suitable for manufacture of tablets, are acceptable. These excipients may be, for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate granulating and disintegrating agents, such as maize starch, or alginic acid; binding agents, such as starch, gelatin or acacia; and lubricating agents, such as magnesium stearate, stearic acid or talc. Tablets may be uncoated or may be coated by known techniques to delay disintegration and adsorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate alone or with a wax may be employed. Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, such as peanut oil, liquid paraffin or olive oil.

The tablets, pills, capsules, troches and the like may contain the following ingredients: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, corn starch and the like; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; and a sweetening agent such as sucrose or saccharin or flavoring agent such as peppermint, methyl salicylate, or orange flavoring may be added. When the dosage unit form is a capsule, it may contain, in addition to material of the above type, a liquid carrier such as a fatty oil. Other dosage unit forms may contain other various materials, which modify the physical form of the dosage unit, for example, as coatings. Thus tablets or pills may be coated with sugar, shellac, or other enteric coating agents. A syrup may contain, in addition to the active polypeptides, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors. Materials used in preparing these various compositions should be pharmaceutically or veterinarially pure and non-toxic in the amounts used.

Aqueous suspensions of the invention contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients include a suspending agent, such as sodium carboxymethylcellulose, methylcellulose,

hydroxypropylethyl cellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethylene oxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol (e.g., polyoxyethylene sorbitol mono-oleate), or a condensation product of ethylene oxide with a partial ester derived from fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan mono-oleate). The aqueous suspension may also contain one or more preservatives such as ethyl or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents, such as sucrose, aspartame, saccharin, or sucralose.

Oil suspensions may be formulated by suspending the active ingredient in a vegetable oil, such as arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oil suspensions may contain a thickening agent, such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an antioxidant such as ascorbic acid.

Dispersible powders and granules of the invention suitable for preparation of an aqueous suspension by the addition of water may be formulated from the active ingredients in admixture with a dispersing, suspending and/or wetting agent, and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those disclosed above. Additional excipients, for example sweetening, flavoring and coloring agents, may also be present.

The compositions of the invention may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil, such as olive oil or arachis oil, a mineral oil, such as liquid paraffin, or a mixture of these. Suitable emulsifying agents include naturally occurring gums, such as gum acacia and gum tragacanth, naturally occurring phosphatides, such as soybean lecithin, esters or partial esters derived from fatty acids and hexitol anhydrides, such as sorbitan mono-oleate, and condensation products of these partial esters with ethylene oxide, such as polyoxyethylene sorbitan mono-oleate. The emulsion may also contain sweetening and flavoring agents.

Syrups and elixirs may be formulated with sweetening agents, such as glycerol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative, a flavoring or a coloring agent.

The compositions of the invention may be in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents, which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, such as a solution of 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water and Ringer's solution, an isotonic sodium chloride. In addition, sterile fixed oils may conventionally be employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may likewise be used in the preparation of injectables. Sterilization may be performed by conventional methods known to those of ordinary skill in the art such as by aseptic filtration, irradiation or terminal sterilization (e.g. autoclaving).

Aqueous formulations (i.e oil-in-water emulsions, syrups, elixers and injectable preparations) may be formulated to achieve the pH of optimum stability. The determination of the optimum pH may be performed by conventional methods known to those of ordinary skill in the art. Suitable buffers may also be used to maintain the pH of the formulation.

The polypeptides of this invention may also be administered in the form of suppositories for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable nonirritating excipient, which is solid at ordinary temperatures but liquid at the rectal temperatures and will therefore melt in the rectum to release the drug. Non-limiting examples of such materials are cocoa butter and polyethylene glycols.

Methods for the production of antibodies directed to a specific peptide or fragment thereof are well known by those of skill in the art. Antibodies can be obtained by injecting an animal with an immunogenic peptide of the invention, or an immunogenic fragment thereof, and recovering the antibodies which are able to complex with said immunogenic peptide or fragment thereof from said animal. Examples of such methods are disclosed in Antibodies, A Laboratory Manual, Harlow and Lane, Cold Spring Harbor Press, 1988, herein incorporated by reference.

Techniques which make it possible to humanize antibodies, either monoclonal or polyclonal, have been described in the following references: Waldmann T. (1991). *Science*. **252**: 1657-1662; Winter G. et al. (1993). *Immunology Today*. **14**(6): 243-246; Carter et al. (1992). *Proc. Natl. Acad. Sci*, USA. **89**: 4285-4289; Singer et al. (1993). *Journal of*

Immunology. **150**(7): 2844-2857, Kipriyanov et al. (2004). *Mol. Biotechnol.* **26**(1):39-60; U.S. Patent No. 6,693,176; U.S. Patent No. 5,225,539; U.S. Patent No. 6,572,857; U.S. Patent No. 6,183,744, each of which are incorporated herein by reference.

An *ipaB* mutant strain of *S. flexneri* expresssing IncA/cya was deposited at C.N.C.M., 25, Rue de Docteur Roux, F-75724, Paris Cedex 15, France, on December 13, 2000, with accession number I-2592.

A bacterial strain containing the vector pUC19cya was deposited at C.N.C.M., 25, Rue de Docteur Roux, F-75724, Paris Cedex 15, France, on December 13, 2000, with accession number I-2593.

An ipaB$^-$ mutant strain of *S. flexneri* designated SF620 was deposited at C.N.C.M., 25, Rue de Docteur Roux, F-75724, Paris Cedex 15, France, on December 13, 2000, with accession number I-2594.

A *E. coli* bacterial strain containing Psi0710 in an expression vector (pQE trisystem, Qiagen) with a carboxy-terminal Histidine tag was deposited at C.N.C.M., 25, Rue de Docteur Roux, F-75724, Paris Cedex 15, France, on February 18, 2003, with accession number I-2974.

EXAMPLES

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

Example 1

Materials and Methods

Bacterial strains and reagents

Strain M90T is the virulent, wild-type strain of *S. flexneri* 5 (Sansonetti *et al.*, 1982). Strains SF401 and SF620 (deposited at C.N.C.M. with accession number I-2594) are derivatives of M90T in which the *mxiD and ipaB* genes, respectively, have been inactivated (Allaoui, A., P.J. Sansonetti, and C. Parsot. (1993). MxiD, an outer membrane protein necessary for the secretion of the Shigella flexneri lpa invasins. *Mol. Microbiol.* **7**: 59-68; Ménard, R., P. Sansonetti and C. Parsot. (1994). The secretion of the Shigella flexneri Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaD. *EMBO J.* **13:** 5293-302). The *E. coli* strain TG1 (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual,

Cold Spring Harbor, New York, 1989) was used for plasmid constructions. *S. flexneri and E. coli* strains were grown in Luria-Bertani (LB). Ampicillin was used at 0.1 mg/ml. Monoclonal antibodies against the calmodulin-dependent adenylate cyclase (Brézin *et al,* 1987). Evidence for the presence of cAMP, cAMP receptor and transcription termination factor Rho in different gram-negative bacteria. *J. Gen. Microbiol.* **131(11)**: 2953-2960) were used. Anti-IpaD antibody was used as described (Ménard, R., P.J. Sansonetti, and C. Parsot. (1993). Horseradish peroxidase-linked secondary antibodies for enhanced chemiluminescence were obtained from Amersham Pharmacia Biotech (Orsay, France). Alkaline phosphatase-linked secondary antibodies for enhanced chemifluorescence were obtained from Pierce (Rockford, IL, USA).

Genomic data

C. pneumoniae protein sequences (CPnXXXX), obtained by sequencing of the CWL029 strain of *C. pneumoniae*, are available at http://chlamydia-www.berkeley.edu:4231/ (chromosome sequence Genbank Accession number: AE001363), see also Kalman et al. (1999) *Nature Genetics*. 21: 385-389. *C. trachomatis* protein sequences (CTXXX), obtained by sequencing serovar D (D/UW-3/Cx) trachoma biovar of *C. trachomatis*, are available at http://chlamydia-www.berkeley.edu:4231/ (chromosome sequence Genbank Accession number: AE001273), see also Stephens et al. (1998) Science. 282: 754-759. *Chlamydophila psittaci* (GPIC strain) sequencing by The Institute for Genomic Research (TIGR, Rockville, MD, Etats-Unis) is not complete but preliminary sequence data was obtained from The Institute for Genomic Research website at http://www.tigr.org. The present Inventors compared *C. pneumoniae* proteins with the translation in the six reading frames of the available sequence using a comparison tool available at http://tigrblast.tigr.org/ufmg/. Herein, the closest protein match from *C. psittaci* to the blasted protein from *C. pneumoniae* (CPnXXXX) is referred to as PsiXXXX.

The entire DNA and amino acid sequences of claimed CPnXXXX, CTXXX, and PsiXXXX designators are attached herewith. The amino acid sequences of the respective proteins used to construct the chimeras are indicated by capital letters.

Construction of recombinant plasmids expressing hybrid proteins

Genomic DNA from *C. pneumoniae* strain TW183 (Kuo et al. (1995) *Clinical Microbiology Reviews*. **8**(4):451-461), serovar D (D/UW-3/Cx) trachoma biovar of *C. trachomatis* and *Chlamydophila psittaci* GPIC strain (American Type Culture Collection,

Virginia, U.S.A.; ATCC No. VR-2282), was prepared from purified bacteria using the RapidPrep Micro Genomic DNA isolation kit (Amersham Pharmacia Biotech). The *C. pneumoniae* DNA fragments used in the examples were amplified by PCR using the strain TW183 as a template. These fragments were sequenced and shown to be more than 99% identical to the sequences of the CWL029 strain. The 5' part of different chlamydial genes, including about 30 nucleotides located upstream from the proposed translation start sites and the first 20 to 99 codons, were amplified by PCR using the primers listed in Table 1. For the Inc/cya constructs, the forward and reverse primers contained additional HindIII *and Xba*I sites, respectively, to allow cloning of the PCR fragments between the HindIII *and Xba*I sites of the puc19cya vector. This vector was constructed by cloning the *Xba*I-*EcoR*I fragment of plasmid pMS109, which carried the *cya* gene of *Bordetella pertussis* (Sory, M.P., and G.R. Cornelis. (1994), between *Xba*I and *Eco*RI sites of the pUC19 vector. In the recombinant plasmids, transcription of the hybrid genes was under the control of the *lac* promoter of the vector. Recombinant plasmids were amplified in *E. coli* TG1 and sequencing checked the sequence of all constructs. Methods of sequencing nucleic acids are known in the art and are described in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York (1989) and Current Protocols in Molecular Biology, Ausebel et al, eds., John Wiley and Sons, Inc., New York (2000).

TABLE I: Oligonucleotides used to construct Inc/cya chimeras.

Protein	**Forward primer**	**Reverse primer**
CPn0009	AGTCAAGCTTATGATTGTACGGACATAGAGACCG (SEQ ID NO: 133)	AGTCTCTAGATGCTTTTCGGACCATAGTC (SEQ ID NO: 250)
CPn0012	AGTCAAGCTTGTAGGTTTATTAAAGGGGATGTACC (SEQ ID NO: 134)	AGTCTCTAGAATCCTCTTCCCAAGGAATCAG (SEQ ID NO: 251)
CPn0026	AGTCAAGCTTGTAGCTGTTCTTTTCAGAGAGCTT (SEQ ID NO: 135)	AGTCTCTAGATCGAATCGATTTGGAAGGAG (SEQ ID NO: 252)
CPn0028	AGTCAAGCTTTACTTTTTGAAGGCTAGTACGTT (SEQ ID NO: 136)	AGTCTCTAGATTCAATAATGCCAGAGCTTTTTC (SEQ ID NO: 253)
CPn0049	AGTCAAGCTTGGAAGGAATATCGTTTACCTGCT (SEQ ID NO: 137)	AGTCTCTAGATTCATCCACCCAAATAGCAC (SEQ ID NO: 254)
CPn0063	AGTCAAGCTTGTAGATGCCCATCCTACCAACAG (SEQ ID NO: 138)	AGTCTCTAGAAGTAAGAGGGAGCCCAGGA (SEQ ID NO: 255)
CPn0066	AGTCAAGCTTTCCCTAAAATGAATAAACTAAGGA (SEQ ID NO: 139)	AGTCTCTAGATGACGGGGGAGGTGTATTAG (SEQ ID NO: 256)
CPn0067	AGTCAAGCTTAAGTGTCATTGAAAAGGTTCAGG (SEQ ID NO: 140)	AGTCTCTAGATAGGAGGCCTTTCGATTGTTT (SEQ ID NO: 257)
CPn0104 (SEQ ID NO: 2)	AGTCAAGCTTGCCAACCAACGGTTAGACGAAA (SEQ ID NO: 141)	AGCTTCTAGATGGAAAGGATACGGATTGG (SEQ ID NO: 258)
CPn0105	AGTCAAGCTTGCGTGCCTTTTAGAAAATTTAGCA (SEQ ID NO: 142)	AGTCTCTAGAGAATGGGGGAATACAAATCA (SEQ ID NO: 259)
CPn0130	AGTCAAGCTTGTAACGACTCCTTCTTTCAACGATT (SEQ ID NO: 143)	AGTCTCTAGAGGCGAGAGAAAGTTTCGTTA (SEQ ID NO: 260)
CPn0132	AGTCAAGCTTCCATTAAGGATCTAAAACAATTT (SEQ ID NO: 144)	AGTCTCTAGACAACTTGTTCATGCGACAG (SEQ ID NO: 261)
CPn0146	AGTCAAGCTTTGTTTGAGATGAATTCGCATTTT (SEQ ID NO: 145)	AGCTTCTAGACGCATCCGAAAGACTTTTCT (SEQ ID NO: 262)

CPn0167	AGTCAAGCTTTTGCATGAATTCGTAAAATAGAAA (SEQ ID NO: 146)	AGTCTCTAGAACGTTCTAAGCCGTAATTCTCG (SEQ ID NO: 263)
CPn0174	AGTCAAGCTTGTAGGGTTTGTGGAGAAAATTGTTA (SEQ ID NO: 147)	AGTCTCTAGATGCGGGGATCGTATAAGCTA (SEQ ID NO: 264)
CPn0175	AGTCAAGCTTCTTCTATTAGCCTGTTTCCAATT (SEQ ID NO: 148)	AGTCTCTAGAAGGTGATTGCTTCCCAAGTCT (SEQ ID NO: 265)
CPn0181	AGTCAAGCTTCTGTTGTTGAATTAATAGCTTCTT (SEQ ID NO: 149)	AGTCTCTAGAATAAGAGTCGAGGGCTTGCAC (SEQ ID NO: 266)
CPn0186	AGTCAAGCTTGTGAGAAAAACAACAATTCTTATCC (SEQ ID NO: 150)	AGTCTCTAGATGTAGGAATACCTGGAGTCGTG (SEQ ID NO: 267)
CPn0206 (SEQ ID NO: 4)	AGTCAAGCTTACGACTCAATTAACAGTGATGCAA (SEQ ID NO: 151)	AGTCTCTAGAGCGCTGAGTAGCGTCGTGTAA (SEQ ID NO: 268)
CPn0210 (SEQ ID NO: 6)	AGTCAAGCTTTCAAGACAATTAGAGAGAAAGAGC (SEQ ID NO: 152)	AGCTTCTAGAGAATTCAAGATGATCCAAACA (SEQ ID NO: 269)
CPn0211	AGTCAAGCTTGTAGGCATTGCTTAAAAATAAAATG (SEQ ID NO: 153)	AGTCTCTAGAAGTCTTATGAAGCAAAGCAG (SEQ ID NO: 270)
CPn0220	AGTCAAGCTTGTAATCGGATTTTAAACCAACTTTT (SEQ ID NO: 154)	AGTCTCTAGAATTTATATGCCACGCCTTCTTTC (SEQ ID NO: 271)
CPn0223	AGTCAAGCTTGTAGGAATTTTTATTACGAGTTTCA (SEQ ID NO: 155)	AGTCTCTAGAGGAGGTTTCCGAGACGATT (SEQ ID NO: 272)
CPn0226	AGTCAAGCTTCAAGAAGCGTTAAGAAAACGAAA (SEQ ID NO: 156)	AGTCTCTAGAATCCCAGTCGTGAGAAGCAT (SEQ ID NO: 273)
CPn0243	AGTCAAGCTTTTATTAAAATTTGTTAAAGGGAGG (SEQ ID NO: 157)	AGTCTCTAGAGAGAAGAATATCTGCAACTAGAGC (SEQ ID NO: 274)
CPn0267	AGTCAAGCTTGTAAACTAATTCGGATTAAAAATGA (SEQ ID NO: 158)	AGTCTCTAGAGGTAACAGTGCATGCGAAAG (SEQ ID NO: 275)
CPn0277	AGTCAAGCTTGAAGGAAAAGAAGTTCTCATAAAG (SEQ ID NO: 159)	AGTCTCTAGACAAAATAGGAATAGCAGCTTGG (SEQ ID NO: 276)
CPn0284	AGTCAAGCTTGTAGCGTCTGGAAAAATTCTGG (SEQ ID NO: 160)	AGTCTCTAGACGGTCTTAAACTACTTTTCAATG (SEQ ID NO: 277)

CPn0287	AGTCAAGCTTGGGTAAGAACACCTTCTAATATG (SEQ ID NO: 161)	AGTCTCTAGAATCAACCACATCTTCTGGACAA (SEQ ID NO: 278)
CPn0291	GACTAAGCTTGTAATCTATTTTTAGATAGGAA (SEQ ID NO: 162)	GACTTCTAGATCCAGGTTTTTCGGAAGCAGA (SEQ ID NO: 279)
CPn0292	AGTCAAGCTTCCCGTAATTACTGTCCGTACAAC (SEQ ID NO: 163)	AGTCTCTAGAAGTAGACGGGAGTTGCTG (SEQ ID NO: 280)
CPn0308	GACTAAGCTTATTATATAGACAGATTAAAAT (SEQ ID NO: 164)	GACTTCTAGACTTAAAAAATACCCAGGAACA (SEQ ID NO: 281)
CPn0330	AGTCAAGCTTAGGGTTTTAAGTCGGTTTGATG (SEQ ID NO: 165)	AGTCTCTAGACCGAATCGCTTCATTCGTAG (SEQ ID NO: 282)
CPn0334	AGTCAAGCTTTCGATAATCATAATTCAAAGCGTA (SEQ ID NO: 166)	AGTCTCTAGAAAGGATCAGATGTGCTTTAGGG (SEQ ID NO: 283)
CPn0357	AGTCAAGCTTTCCATGAATTCTAAAATGATTAGG (SEQ ID NO: 167)	AGTCTCTAGACGAAAATTGGGTAGCCTGAC (SEQ ID NO: 284)
CPn0365	AGTCAAGCTTAATTTAAAGGAACTCAGGTGAA (SEQ ID NO: 168)	AGTCTCTAGAATCAAAGTTGCTGCAGGATT (SEQ ID NO: 285)
CPn0374	AGTCAAGCTTGTAACATGGGGTATGGAAAGACC (SEQ ID NO: 169)	AGTCTCTAGACTCGGCATCCTTTTGTTTTG (SEQ ID NO: 286)
CPn0379	AGTCAAGCTTGTAAGCACCTGCTCTCGAATACA (SEQ ID NO: 170)	AGTCTCTAGATTCTTCTTGGTGCCTGCTAA (SEQ ID NO: 287)
CPn0399 (SEQ ID NO: 8)	AGTCAAGCTTGTAACGGTCTCTTTGCTTGTTTTT (SEQ ID NO: 171)	AGTCTCTAGAAGTGCAGCTGGATAGGGTTG (SEQ ID NO: 288)
CPn0405 (SEQ ID NO: 10)	AGTCAAGCTTGTAACGATCCGAACTTATCGTAGT (SEQ ID NO: 172)	AGTCTCTAGATCCGGATGCGGTAGTAGTTG (SEQ ID NO: 289)
CPn0443 (SEQ ID NO: 12)	AGTCAAGCTTGTAAATTCATGGGCCTAATGATAA (SEQ ID NO: 173)	AGTCTCTAGATAACCCTGTGGATGACGTTTT (SEQ ID NO: 290)
CPn0480 (SEQ ID NO: 14)	AGTCAAGCTTGTAATTGGGGAATCTCTGGGAGAC (SEQ ID NO: 174)	AGTCTCTAGAGGGACTAACGACCTCAGCAC (SEQ ID NO: 291)
CPn0489 (SEQ ID NO: 16)	AGTCAAGCTTGTAATGGAGGATTGGCTAAGGA (SEQ ID NO: 175)	AGTCTCTAGAAACACCACCGACATCACAAA (SEQ ID NO: 292)

CPn0490 (SEQ ID NO: 18)	AGTCAAGCTTGTAAGAGGGACCTTACCTATTGCTT (SEQ ID NO: 176)	AGTCTCTAGAAATGAGGACCTCTCCTTCGT (SEQ ID NO: 293)
CPn0497 (SEQ ID NO: 20)	AGTCAAGCTTGCGGAATGGTTAAAGGAAACG (SEQ ID NO: 177)	AGTCTCTAGATTGTCCATCAAAGCCTACAAT (SEQ ID NO: 294)
CPn0522 (SEQ ID NO: 22)	AGTCAAGCTTGTAAAACCAGCCTAGCCTTCAAA (SEQ ID NO: 178)	AGTCTCTAGAGCTTTTTGCATAGGGGAAG (SEQ ID NO: 295)
CPn0556 (SEQ ID NO: 24)	AGTCAAGCTTGTAACCTGCTTCTTTAGGAACTAC (SEQ ID NO: 179)	AGTCTCTAGATTGTAGACCAGCGGTGACACT (SEQ ID NO: 296)
CPn0582 (SEQ ID NO: 26)	AGTCAAGCTTGTAAAATTCAGCACAGGCTTGTAA (SEQ ID NO: 180)	AGTCTCTAGAGCGTTCTGGAGTGACGAAAC (SEQ ID NO: 297)
CPn0585	GACTAAGCTTGTAAATTGGAGATTGTAGTAGC (SEQ ID NO: 181)	GACTTCTAGAAACAATTGTATGATTCCATCC (SEQ ID NO: 298)
CPn0588 (SEQ ID NO: 28)	ATGCAAGCTTACGAGCAAGAGCATAAAATCCA (SEQ ID NO: 182)	ATGCTCTAGACCTTTGGCGGACTTTTTCTT (SEQ ID NO: 299)
CPn0595 (SEQ ID NO: 30)	AGTCAAGCTTGAATTGCTAACAGACACTAAAGG (SEQ ID NO: 183)	AGTCTCTAGATACCCATTCTTGACCGGAAA (SEQ ID NO: 300)
CPn0648	AGTCAAGCTTGTTTTAAAAAGCCTTGTAAGGAGGT (SEQ ID NO: 184)	AGTCTCTAGACTGAGCAAAGGAGCTGACAG (SEQ ID NO: 301)
CPn0671 (SEQ ID NO: 32)	AGTCAAGCTTGTAAGAATTACCATAAATCAGAGGAA (SEQ ID NO: 185)	AGTCTCTAGAAGCATCAGTGCAATGAGGATAA (SEQ ID NO: 302)
CPn0673 (SEQ ID NO: 34)	AGTCAAGCTTGTAACAGAAGGAAAAGCATACCACTG (SEQ ID NO: 186)	AGTCTCTAGATCCAAAACGATCCTGTTCC (SEQ ID NO: 303)
CPn0681 (SEQ ID NO: 36)	AGTCAAGCTTGCCATAAGTCGTTTACAAGATCG (SEQ ID NO: 187)	AGTCTCTAGATTCCACACAAGAGACCACCA (SEQ ID NO: 304)
CPn0705	AGTCAAGCTTGTGAATCAGGGGGAAGCTAGT (SEQ ID NO: 188)	AGTCTCTAGATCGTGTTTGCTTTCCTTCCA (SEQ ID NO: 305)
CPn0710	AGTCAAGCTTCGCAAGATGATATAAAGGTCCA (SEQ ID NO: 189)	AGTCTCTAGAGACAGTGCCTTGTGTTGATG (SEQ ID NO: 306)
CPn0711	AGTCAAGCTTTAAATAACCAAGTATTGGGGTTTA (SEQ ID NO: 190)	AGTCTCTAGATCGAAGCAGCGCATGTAACT (SEQ ID NO: 307)

CPn0712 (SEQ ID NO: 38)	AGTCAAGCTTGTAGACATGGCTGTCAGATCTTGG (SEQ ID NO: 191)	AGTCTCTAGAAGAGTCGCGTCCTATAGACCA (SEQ ID NO: 308)
CPn0720 (SEQ ID NO: 40)	AGTCAAGCTTCTCTTTTTAAAGACAACGCGAAT (SEQ ID NO: 192)	AGCTTCTAGACGTGTGAGTCCCCTGAACTT (SEQ ID NO: 309)
CPn0725 (SEQ ID NO: 42)	AGTCAAGCTTGTAATTAATCTTGCGGAGATGTTGG (SEQ ID NO: 193)	AGTCTCTAGACTCTGTAGTCAGCTGGTCGTTG (SEQ ID NO: 310)
CPn0729 (SEQ ID NO: 44)	AGTCAAGCTTGTAAGCCTGCTTGGTCTTCTGTT (SEQ ID NO: 194)	AGTCTCTAGACGAGAGAGGAAGTTCAGCGTA (SEQ ID NO: 311)
CPn0746 (SEQ ID NO: 46)	ATGCAAGCTTGTAAGGGAAGATGTCCGTTCAGTT (SEQ ID NO: 195)	ATGCTCTAGAAGCTCCCTTAACGACTTCTGG (SEQ ID NO: 312)
CPn0755 (SEQ ID NO: 48)	AGTCAAGCTTATGGCTATGAAGAGCAATTTATTC (SEQ ID NO: 196)	AGTCTCTAGAAGGGTAACACACTAGGGGAAGA (SEQ ID NO: 313)
CPn0761 (SEQ ID NO: 50)	AGTCAAGCTTGTAACTCCCGCACCAACCTC (SEQ ID NO: 197)	AGTCTCTAGATCCTTCAGACCAACGCTGATA (SEQ ID NO: 314)
CPn0764 (SEQ ID NO: 52)	AGTCAAGCTTGTAATCTGGTTAACACATGCTGAGG (SEQ ID NO: 198)	AGTCTCTAGATGACAGGCCGTTTCTATCAA (SEQ ID NO: 315)
CPn0770 (SEQ ID NO: 54)	AGTCAAGCTTGTAAGAGCCGAGCATAGATAGAAAC (SEQ ID NO: 199)	AGTCTCTAGAGCCTGCAATCAATCCCTGT (SEQ ID NO: 316)
CPn0774 (SEQ ID NO: 56)	AGTCAAGCTTTTAATAAGCTGAATAAACCAATGA (SEQ ID NO: 200)	AGTCTCTAGATTCTGCGGAAGGAAGCTGT (SEQ ID NO: 317)
CPn0792 (SEQ ID NO: 58)	AGTCAAGCTTGTAACATGACGACATCACCTTATT (SEQ ID NO: 201)	AGTCTCTAGAAGCGGCAGAAAATGAGAAAA (SEQ ID NO: 318)
CPn0820	AGTCAAGCTTGTAAGTCAGGAAGTCCGTGGTGAT (SEQ ID NO: 202)	AGTCTCTAGACAAAGCAATTAGAGTGAACGACA (SEQ ID NO: 319)
CPn0821	AGTCAAGCTTGTAATTTGCGGTTGGGAAATAA (SEQ ID NO: 203)	AGTCTCTAGAAGCACAGGCACAACGTTTAC (SEQ ID NO: 320)
CPn0829	AGTCAAGCTTGTAACCCTTGCCTCTATTTTGAGA (SEQ ID NO: 204)	AGTCTCTAGACGGTACCAAGGGCCATTTTG (SEQ ID NO: 321)
CPn0853 (SEQ ID NO: 60)	AGTCAAGCTTGTAATTGCGAGAAGGATTAAATCTTAG (SEQ ID NO: 205)	AGTCTCTAGAAAGATGTCGGGAAAGTGTCG (SEQ ID NO: 322)

CPn0859 (SEQ ID NO: 62)	AGTCAAGCTTATCTCAAACATCAAGTGCTGAA (SEQ ID NO: 206)	AGTCTCTAGATTGGCTTGCCTTATCATTCG (SEQ ID NO: 323)
CPn0879 (SEQ ID NO: 64)	AGTCAAGCTTGTAAGCCTCGTCAAATCCTGA (SEQ ID NO: 207)	AGTCTCTAGAGACGACTTGCTTGCTCACT (SEQ ID NO: 324)
CPn0906 (SEQ ID NO: 66)	AGTCAAGCTTTCTTCAGGAACTTTAAAAACA (SEQ ID NO: 208)	AGTCTCTAGATGCTGCGACACGAATTTCTA (SEQ ID NO: 325)
CPn0939 (SEQ ID NO: 68)	AGTCAAGCTTTAATAGAGCTCATTGGAGGAGGA (SEQ ID NO: 209)	AGTCTCTAGAGAGGAGGGAAACACCGTTAAT (SEQ ID NO: 326)
CPn1002 (SEQ ID NO: 70)	AGTCAAGCTTGTAATGTAGAAAAGCGCAGAGAGAAA (SEQ ID NO: 210)	AGTCTCTAGACCCTTCCAATTGAGGAAAA (SEQ ID NO: 327)
CPn1005 (SEQ ID NO: 72)	AGTCAAGCTTGTAATCGAAAACCAGTTAGGTTGAA (SEQ ID NO: 211)	AGTCTCTAGATGCAATGATATCGTCAGCAG (SEQ ID NO: 328)
CPn1007 (SEQ ID NO: 74)	AGTCAAGCTTGTAATGGGTCCTAATGGAGGCTTT (SEQ ID NO: 212)	AGTCTCTAGACACCTGGATTTTGCCTTCAG (SEQ ID NO: 329)
CPn1016	AGTCAAGCTTGACCTCCTTGTAACCATTCTCG (SEQ ID NO: 213)	AGTCTCTAGACTTCCATGGTAAAGGAGCA (SEQ ID NO: 330)
CPn1019 (SEQ ID NO: 76)	AGTCAAGCTTTCCTTAAGAGGATAAATCCACAG (SEQ ID NO: 214)	AGTCTCTAGATTCTGCAGCAACAACAGCTT (SEQ ID NO: 331)
CPn1020 (SEQ ID NO: 78)	AGTCAAGCTTTTCGAAAACATAAATAATGTGGA (SEQ ID NO: 215)	AGTCTCTAGAAGCTTCTTGAGGCGCTACAG (SEQ ID NO: 332)
CPn1022	AGTCAAGCTTGTGCAGGAAAATGATGTTTTAGC (SEQ ID NO: 216)	AGTCTCTAGATCCAGAGGCTGTGGAAACTGT (SEQ ID NO: 333)
CPn1027	AGTCAAGCTTCAGCCTGGTTTCGTAATTTT (SEQ ID NO: 217)	AGTCTCTAGAAGCATGAGGCTGTATGAGG (SEQ ID NO: 334)
CPn1032 (SEQ ID NO: 80)	AGTCAAGCTTGTAAACCACACCAATATTATTTAGGA (SEQ ID NO: 218)	AGTCTCTAGATGCTTGTAGAAGAGCAGAATCG (SEQ ID NO: 335)
CPn1058 (SEQ ID NO: 82)	AGTCAAGCTTGTAACCTGGGCAGGTCAAAATCTA (SEQ ID NO: 219)	AGTCTCTAGACCCAGATGAAGTCACTGGAAC (SEQ ID NO: 336)
CT053	AGTCAAGCTTGTAATGGGTTTCCGGTTTCAATCA (SEQ ID NO: 220)	AGTCTCTAGAACGGATTTCTTCCTGGTGTCT (SEQ ID NO: 337)

CT083	AGTCAAGCTTGTAAGCCAAGAGCAGGATAAAACGA (SEQ ID NO: 221)	AGTCTCTAGATGATATGCTCCCATTCTTTCG (SEQ ID NO: 338)
CT387 (SEQ ID NO: 84)	AGTCAAGCTTGTAAATTTTTCCTATAAGCTGGTTC (SEQ ID NO: 222)	AGTCTCTAGATCCTTCGTATACGCCAGTACC (SEQ ID NO: 339)
CT476 (SEQ ID NO: 86)	AGTCAAGCTTTTGATAAGAAATTAGCCAACATAGG (SEQ ID NO: 223)	AGTCTCTAGAGCATCCACGTTTGTTCCATT (SEQ ID NO: 340)
CT529	AGTCAAGCTTTTCGGTTTAAGTAATAGAAGTGG (SEQ ID NO: 224)	AGTCTCTAGAAGCATTCCCTGTACCAGACC (SEQ ID NO: 341)
CT550 (SEQ ID NO: 88)	AGTCAAGCTTGTAAGACGGTGCCTTCAATAACAAA (SEQ ID NO: 225)	AGTCTCTAGAAGCCGAACGAGCTAAGACAT (SEQ ID NO: 342)
CT606.1 (SEQ ID NO: 90)	AGTCAAGCTTGTAAAAACAGGGAAAGACGATGA (SEQ ID NO: 226)	AGTCTCTAGAGACCAAAGCTCCCTCTTGAA (SEQ ID NO: 343)
CT610 (SEQ ID NO: 92)	AGTCAAGCTTGTAAGCGGTTACGTGGAGTCAA (SEQ ID NO: 227)	AGTCTCTAGAGGCATACGCCTGTAATTGCT (SEQ ID NO: 344)
CT642 (SEQ ID NO: 94)	AGTCAAGCTTGTAATCCGGAGCCTTTCTCCTAT (SEQ ID NO: 228)	AGTCTCTAGATTCTTCAGGGCCAGCAA (SEQ ID NO: 345)
CT652.1 (SEQ ID NO: 96)	AGTCAAGCTTGTAAAGATGAATGATTCCAGAAAG (SEQ ID NO: 229)	AGTCTCTAGAAGGAAAGCCTATCGCACACA (SEQ ID NO: 346)
CT664 (SEQ ID NO: 98)	AGTCAAGCTTGGACTAAGTAAAACGGAGCAGGA (SEQ ID NO: 230)	AGTCTCTAGAAGACCAACTCGTCCCATTTTC (SEQ ID NO: 347)
CT665	AGTCAAGCTTTTCGAGGTTTATTTAATTCTTCCA (SEQ ID NO: 231)	AGTCTCTAGAGTGGGCTTTGGTTACATCGT (SEQ ID NO: 348)
CT666	AGTCAAGCTTACGTATCAACCGTAAAATGGTG (SEQ ID NO: 232)	AGTCTCTAGAAGTTCCTTGCGTGGATGTTT (SEQ ID NO: 349)
CT671	AGTCAAGCTTGTAAGCAAAAACAACGGGAATC (SEQ ID NO: 233)	AGTCTCTAGACATCCGATCTGCTTTCTCTTG (SEQ ID NO: 350)
CT718 (SEQ ID NO: 100)	AGTCAAGCTTGTAAATGAAGAGTGCTGTGTTAAAGT (SEQ ID NO: 234)	AGTCTCTAGAATGGGAGAGAGCTTCTTGCT (SEQ ID NO: 351)
CT763 (SEQ ID NO: 102)	AGTCAAGCTTGTAACACAATCTTGGGCAAGAGAC (SEQ ID NO: 235)	AGTCTCTAGAGATCTCCAGCTTAAGGGTTGC (SEQ ID NO: 352)

CT845 (SEQ ID NO: 104)	AGTCAAGCTTGTAAGAGAAGAGAAAGGCTAAGGATG (SEQ ID NO: 236)	AGTCTCTAGAAGGGTTCTCCTCAAGTTCATC (SEQ ID NO: 353)
CT848 (SEQ ID NO: 106)	AGTCAAGCTTATAAACCCCTGTCTTAACCCA (SEQ ID NO: 237)	AGTCTCTAGACGTTGCAGAGTTCGTTGTTC (SEQ ID NO: 354)
CT863	AGTCAAGCTTTCCTTTCTAAAAGGCCTGGA (SEQ ID NO: 238)	AGCTTCTAGACAGAACAACGTCTCCTACGC (SEQ ID NO: 355)
Psi0330 (SEQ ID NO: 108)	AGTCAAGCTTGCAAATCTAAGGAGTTAGGTTAGGG (SEQ ID NO: 239)	AGCTTCTAGAAGCCAAACGCATAGCTTCAT (SEQ ID NO: 356)
Psi0379 (SEQ ID NO: 110)	AGTCAAGCTTGTAATACAGGATGGTGCCCACT (SEQ ID NO: 240)	AGTCTCTAGATAGAAGTTGCAGGCGTTCCT (SEQ ID NO: 357)
Psi0595 (SEQ ID NO: 112)	AGTCAAGCTTGCGCAAATAAACGATACAA (SEQ ID NO: 241)	AGTCTCTAGATCCGTAGTTGTTACGGAAGGTT (SEQ ID NO: 358)
Psi0648 (SEQ ID NO: 114)	AGTCAAGCTTCATTGTGTTATAATTGCAGGCTA (SEQ ID NO: 242)	AGTCTCTAGATTTCTTGGCTCCCTGCATAG (SEQ ID NO: 359)
Psi0671 (SEQ ID NO: 116)	AGTCAAGCTTTGACGCCCCTTAAAATAAAGA (SEQ ID NO: 243)	AGTCTCTAGACGCAGAATGGGATAGGACAT (SEQ ID NO: 360)
Psi0710 (SEQ ID NO: 120)	AGTCAAGCTTCCGCAAAATGGTTTAACAGA (SEQ ID NO: 244)	AGTCTCTAGATTGCACACCTTGGACGTACT (SEQ ID NO: 361)
Psi0761 (SEQ ID NO: 122)	AGTCAAGCTTGTAAGCTTGGGAATCTACACATTTTT (SEQ ID NO: 245)	AGTCTCTAGATTTCGTTAATTCTCCCTTAGACCA (SEQ ID NO: 362)
Psi0774 (SEQ ID NO: 124)	AGTCAAGCTTGTAAAAGCTGAATAAGCCCATGA (SEQ ID NO: 246)	AGTCTCTAGAAAGTTTCTGTGTCGCTACGG (SEQ ID NO: 363)
Psi1002 (SEQ ID NO: 126)	AGTCAAGCTTGTAACCGTGAGGAGTCTGAACAA (SEQ ID NO: 247)	AGTCTCTAGAAGAAAAATCCATGGGCTGTAA (SEQ ID NO: 364)
Psi1005 (SEQ ID NO: 128)	AGTCAAGCTTTTAATCGAAGAAAATAGGTAGGAAA (SEQ ID NO: 248)	AGTCTCTAGATTCATCGGCTGTTGCTGTAT (SEQ ID NO: 365)
Psi1022 (SEQ ID NO: 130)	AGTCAAGCTTGTAACGCTTCTACATCCCCATAATT (SEQ ID NO: 249)	AGTCTCTAGAGAATACGGAAAGCGCAACAT (SEQ ID NO: 366)

Transformation of S. flexneri ipaB strain and test of secretion via the type III pathway

Shigella flexneri (*ipaB* strain, I-2594) were transformed with the constructs made by the process above and colonies expressing a CPn/cya, CT/cya, or Psi/cya chimera were isolated on plates containing the Congo Red dye. Only red colonies, indicative of a constitutive high level of type III secretion, were considered. Such colonies were picked on a LB plate and incubated for 6 hours at 37°C. A polyvinylidene fluoride membrane (Immobilon-P, Millipore) was deposited on top of the colonies, and the plate was incubated overnight at 37°C. The membrane was briefly incubated in ethanol and then processed for Western blotting using anti-cyclase antibody described (Subtil et al (2001) Mol. Microbiol. 39:792-800). Revelation was done by enhanced chemifluorescence (Amersham). The signal for colonies in which the CPn/cya, CT/cya, or Psi/cya chimeras were not secreted was restricted to the area of the membrane that had been in contact with the colonies. The signal for colonies in which the CPn/cya, CT/cya, or Psi/cya chimeras were secreted appeared as a halo around the area of the membrane that had been in contact with the colonies (see Figure 1).

Negative controls-

Chlamydial proteins that are homologous to cytosolic proteins in other bacteria species are not expected to be secreted during *Chlamydia* infection and therefore should not be positive for secretion in the present assay. To validate the specificity of this assay, the Inventors constructed 9 chimeras between putative cytosolic chlamydial proteins and the cya reporter molecule. None of these chimeras were secreted by the *ipaB* strain of *S. flexneri*. This result shows that the test developed by the present Inventors is specific for detecting secreted proteins.

The 9 chimeras that were constructed used the amino terminal region of CPn0032, CPn0089, CPn0103, CPn0115, CPn0184, CPn0202, CPn0280, CPn0320, CPn0402.

Negative results-

Most of the 280 chimera that the Inventors tested were not secreted by the *ipaB* strain. This result was expected since in other bacteria species such as *Shigella*, type III secretion allows for the secretion of only a limited number of proteins (up to date about 25 have been identified for *S. flexneri* (Buchrieser C., P. Glaser, C. Rusniok, H. Nedjari, H. d'Hauteville, F. Kunst, P. Sansonetti, and C. Parsot. (2000). The virulence plasmid pWR100 and the

repertoire of proteins secreted by the type III secretion apparatus of *Shigella flexneri*. *Mol. Microbiol.* **38**(4): 760-771).

Examples of Chlamydial proteins for which the corresponding chimera were not secreted in the *ipaB* strain are:

CPn0010, CPn0011, CPn0034, CPn0036, CPn0042, CPn0046, CPn0050, CPn0051, CPn0055, CPn0062, CPn0070, CPn0071, CPn0084, CPn0085, CPn0087, CPn0089, CPn0093, CPn0095, CPn0099, CPn0100.

Detecting secreted proteins by the type III secretion system by *Shigella flexneri*, the secretion of chimeric forms (consisting of the amino-terminal domain of these proteins fused with the Cya reporter) of *Chlamydia* spp. proteins was determined. The secreted chimeric proteins can be classified in 5 categories:

Category 1- Corresponding chimeras from *C. pneumoniae, C. trachomatis,* and *C. psittaci* that are homologous and are secreted. Sequence analysis has shown that these three species are rather distant, and, as a consequence, the percentage of amino acid identity between homologous proteins is generally low. Consequently, the chimeras constructed from homologous proteins share little identity in their amino terminal region, which is the region containing the putative secretion signal. Therefore, the identification that the three homologous chimeras (one from each of the *Chlamydia* species above) are secreted strongly supports that the corresponding proteins are secreted during *Chlamydia* infection.

Proteins of *C. pneumoniae* (CPnXXXX), *C. trachomatis* (CTXXX), and *C. psittaci* (PsiXXXX) which are part of this category are:

CPn0330 ; CT083 ; Psi0330 ; CPn0379 ; CT053 ; Psi0379 ; CPn0595 ; CT476 ; Psi0595 ; CPn0648 ; CT529 ; Psi0648 ; CPn 0671 ; CT550 ; Psi0671 ; CPn0710 ; CT666 ; Psi0710 ; CPn0761 ; CT610 ; Psi0761 ; CPn0774 ; CT606.1 ; Psi0774 ; CPn1002 ; CT845 ; Psi1002 ; CPn1005 ; CT848 ; Psi1005 ; CPn1022 ; CT863 ; Psi1022.

Category 2- Proteins of *C. pneumoniae* and *C. trachomatis* which are homologous and for which the 2 corresponding chimeras are secreted. For the same reasons as explained above in the case of category 1, these results support that these proteins are likely secreted during *Chlamydia* infection.

Proteins of *C. pneumoniae* (CPnXXXX) and *C. trachomatis* (CTXXX) which are part of this category are:

CPn0490 ; CT387 ; CPn0705 ; CT671 ; CPn0711 ; CT665 ; CPn0712 ; CT664 ; CPn0725 ; CT652.1 ; CPn0770 ; CT642 ; CPn0859 ; CT718 ; CPn0906 ; CT763.

Category 3- *C. pneumoniae* proteins that are secreted, which have no homolog in *C. trachomatis* or *C. psittaci*. They may be important proteins, being specific of *C. pneumoniae*.

C. pneumoniae proteins (CPnXXXX) which are part of this category are:
CPn0009 ; CPn0012 ; CPn0028 ; CPn0049; CPn0063 ; CPn0066 ; CPn0067 ; CPn0130 ; CPn0132 ; CPn0146 ; CPn0167 ; CPn0174 ; CPn0175 ; CPn0181 ; CPn0210; CPn0211 ; CPn0220 ; CPn0223 ; CPn0226 ; CPn0243 ; CPn0267 ; CPn0277 ; CPn0284 ; CPn0357 ; CPn0365 ; CPn0585 ; CPn0829 ; CPn1027.

Category 4- *C. pneumoniae* proteins that have a homolog in at least one of the other 2 sequenced *Chlamydia* species and for which secretion of the corresponding homologous chimera have not been tested for technical or scientific reason (for example when the percentage of identity between the homologous proteins was very high).

C. pneumoniae (CPnXXXX) proteins that have a homolog in at least one of the other 2 sequenced *Chlamydia* species and for which secretion of the corresponding homologous chimeras have not been tested, and are part of this category are:
CPn0026 ; CPn0104 ; CPn0186 ; CPn0206 ; CPn0291 ; CPn0292 ; CPn0405 ; CPn0443 ; CPn0480 ; CPn0489 ; CPn0556 ; CPn0673 ; CPn0681 ; CPn0720 ; CPn0746 ; CPn0853 ; CPn0879 ; CPn0939 ; CPn1019 ; CPn1020 ; CPn1032.

Category 5- *C. pneumoniae* proteins that have homologs in the other 2 sequenced *Chlamydia* species and for which secretion of the corresponding homologous chimeras have either not been tested or are negative or undetermined. Although these results support that most proteins in this category are secreted by *Chlamydia* during infection, the Inventors regard them as somewhat less strong candidates than the proteins classified in the other 4 categories, until more data are obtained (see for example the result with Psi1058 below).

C. pneumoniae (CPnXXXX) proteins that have homologs in the other 2 sequenced *Chlamydia* species and for which secretion of the corresponding homologous chimeras have either not been tested or are negative or undetermined, and are part of this category are:
CPn0105 ; CPn0287 ; CPn0334 ; CPn0374 ; CPn0399 ; CPn0497 ; CPn0522 ; CPn0582 ; CPn0588 ; CPn0729 ; CPn0755 ; CPn0764 ; CPn0792 ; CPn0820 ; CPn0821 ; CPn1007 ; CPn1016 ; CPn1058.

Example 2

Chlamydial genes were cloned by PCR for expression of full-length chlamydial proteins with a carboxy-terminal Histidine-tag. The forward and reverse primers contained additional *NcoI* and *KpnI* sites, respectively, to allow cloning of the PCR fragments between the *NcoI* and *KpnI* sites of the pQE-TriSystem expression vector (Qiagen). Sequences of the primers are given in Table II. Recombinant plasmids were amplified in *E. coli* TG1 and the sequences were checked by sequencing.

Plasmids were used to transform the strains SF401 and SF620 which are derivatives of M90T in which the *mxiD* and *ipaB* genes, respectively, have been inactivated (Allaoui *et al.*, 1993; Ménard *et al.*, 1993). Transformed colonies were isolated on plates containing 100 µg/ml ampicillin.

Analysis of secreted proteins was performed as previously described (Allaoui *et al.*, 1993). Briefly, 1 ml of an overnight culture at 30 °C was inoculated in 30 ml of LB and incubated at 37 °C for 3 h. Bacteria were harvested by centrifugation and the culture supernatant was filtered through 0.22 µm filters. Proteins present in 25 ml of the filtrates were precipitated by the addition of 1/10 (v/v) trichloroacetic acid, and resuspended in 0.5 ml of sample buffer for electrophoresis. Equal volumes of samples of the bacterial pellet and culture supernatants, the latter being concentrated 25-fold as compared to the pellet, were analyzed by electrophoresis in 12% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE). After electrophoresis, proteins were transferred on a PVDF membrane (Millipore Corporation, Bedford, MA), and the membrane was used for blotting with anti-His antibody (H-15, sc-803, Santa Cruz Biotechnology). Western blotting and revelation were performed by enhanced chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Table II

Protein	Forward primer	Reverse primer
CPn0175	ATCGTACCATGGAGCAACCCAATTGTG (SEQ ID NO: 367)	AGTCGGTACCAGCTTCCTTAACTTCGCTGAG (SEQ ID NO: 373)
Psi0705	ATCGTACCATGGAATTAAATAAAACATCCGAGTCTTTG (SEQ ID NO: 368)	AGTCGGTACCTTGGTTTTGTTCTTGATCTTGC (SEQ ID NO: 374)
Psi0725	ATCGTACCATGGTTCTCGCTTCTTGTCTTCTTG (SEQ ID NO: 369)	AGTCGGTACCATCTAAGAAATCATCTTCTGTAAGGAC (SEQ ID NO: 375)
Psi0774	ATCGTACCATGGATGAAGGTATTCAAACCGTTTCT (SEQ ID NO: 370)	AGTCGGTACCGTCTCTAGGAACTAGCGTTTCCA (SEQ ID NO: 376)
Psi1005	ATCGTACCATGGGGTTCTCTTCTCCAGCACTTC (SEQ ID NO: 371)	AGTCGGTACCAATGTTTGCTATCAAACTTACAATT (SEQ ID NO: 377)
Psi1058	ATCGTACCATGGAGAACAAAACACTAAGCGTTTTCT (SEQ ID NO: 372)	AGTCGGTACCCAATGAAAATAGAGGCGGA (SEQ ID NO: 378)

The present inventors have shown that CPn0175, Psi0705, Psi0725, Psi0774, Psi1005 and Psi1058, expressed as full-length proteins with a C-terminal histidine tag, are found in the supernatant of the transformed *ipaB* cultures. When they are expressed in the *mxiD* strain, which is deficient for type III secretion, these proteins are absent from the culture supernatants (see Figure 2). These results confirm that CPn0175, Psi0705, Psi0725, Psi0774, Psi1005 and Psi1058 are secreted by a type III mechanism in *S. flexneri*.

Example 3

Proteins Psi0705 and Psi0710 were expressed in *E. coli* with a carboxy-terminal Histidine tag and purified by standard divalent metal column chromatography methods according to the manufacturer's instructions (Qiagen). Purified proteins were used to immunize rabbits and specific antibodies against Psi0705 and Psi0710 were obtained. These antibodies were used to study Psi0705 and Psi0710 localization during *Chlamydia* infection.

A: By immunofluorescence, the present inventors determined that Psi0710 is associated with the membrane of the inclusion in HeLa cells infected with *C. psittaci* GPIC strain (Figure 4). As a control, the present inventors determined that the pre-immune serum did not label infected cells, and that antibodies against the Major Outer Membrane Protein of Chlamydia did not label the membrane of the inclusion.

B: By Western Blot, the present inventors determined that Psi0705 is associated with the cytosolic fraction of HeLa cells infected with *C. psittaci* GPIC strain (Figure 3). The same result was obtained using antibodies specific for Psi0710. As a control, the present inventors determined that the Major Outer Membrane Protein of Chlamydia was not found in the cytosolic fraction of HeLa cells infected with *C. psittaci* GPIC strain.

These experiments demonstrate that Psi0705 and Psi0710 are secreted by *Chlamydia* during infection. Therefore, these data fully confirm the inventive approach to identify such proteins using heterologous type III secretion as described in the present invention.

Obviously, numerous modifications and variations on the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.